Substance P promotes diabetic corneal epithelial wound healing through molecular mechanisms mediated via the neurokinin-1 receptor

Running title: Mechanisms of SP on diabetic corneal wound healing

Lingling Yang¹, Guohu Di¹, Xia Qi¹, Mingli Qu¹, Yao Wang¹, Haoyun Duan¹, Patrik Danielson², Lixin Xie¹*, Qingjun Zhou¹*

¹ State Key Laboratory Cultivation Base, Shandong Provincial Key Laboratory of Ophthalmology, Shandong Eye Institute, Shandong Academy of Medical Sciences, Qingdao, China.

² Department of Integrative Medical Biology, Anatomy, Umeå University, Umeå, Sweden

# Contribute equally to this study

* Correspondence: Qingjun Zhou & Lixin Xie

Address: Shandong Eye Institute, 5 Yan’erdao Road, Qingdao, 266071, China.

Email: qjzhou2000@hotmail.com (Zhou Q); lixin_xie@hotmail.com (Xie L).

Tel: 86-532-8589-9270;

Fax: 86-532-8589-1110

Word count: 3993; number of Figures: 7; supplement Figures/Tables: 5/2
Abstract

Substance P (SP) is a neuropeptide, predominantly released from sensory nerve fibers, with a potentially protective role in diabetic corneal epithelial wound healing. However, the molecular mechanism remains unclear. We investigated the protective mechanism of SP against hyperglycemia-induced corneal epithelial wound healing defects, using type 1 diabetic mice and high glucose-treated corneal epithelial cells. Hyperglycemia induced delayed corneal epithelial wound healing, accompanied with attenuated corneal sensation, mitochondrial dysfunction, and impairments of Akt-, EGFR-, and Sirt1-activation, as well as decreased reactive oxygen species (ROS) scavenging capacity. However, SP application promoted the epithelial wound healing, the recovery of corneal sensation, the improvement of mitochondrial function, and the reactivation of Akt, EGFR and Sirt1, as well as increased ROS scavenging capacity, in both diabetic mouse corneal epithelium and high glucose-treated corneal epithelial cells. The promotion of SP on diabetic corneal epithelial healing was completely abolished by a NK-1 receptor antagonist. Moreover, the subconjunctival injection of NK-1 receptor antagonist also caused diabetic corneal pathological changes in normal mice. In conclusion, the results suggest that SP-NK-1 receptor signaling plays a critical role in the maintenance of corneal epithelium homeostasis, and that SP signaling through the NK-1 receptor contributes to the promotion of diabetic corneal epithelial wound healing by rescued activation of Akt, EGFR, and Sirt1, improvement of mitochondrial function, and increased ROS scavenging capacity.

Keywords: Corneal epithelium; Diabetes; Substance P; Neurokinin-1 receptor
INTRODUCTION

Among various pathological conditions by diabetes mellitus, ocular complications have been a leading cause of blindness in the world, including diabetic retinopathy, cataract, and various ocular surface disorders (1). The most recognized diabetic changes in the cornea, i.e. clinical diabetic keratopathy, include impaired corneal sensation, superficial punctate keratitis, and persistent corneal epithelium defects (2). The uncontrolled impairment of corneal wound healing increases the susceptibility of corneal ulcer, microbial keratitis, and even perforation. Diabetic corneal pathology always exhibits epithelial basement membrane abnormalities, reduced hemidesmosome density, and delayed wound healing (3; 4). However, hyperglycemia also directly impairs the cellular metabolism and causes abnormal changes of corneal epithelium, such as Akt-, epidermal growth factor receptor (EGFR)- and Sirt1-mediated cell responses to environmental challenges (5-7). Moreover, excess oxidative stress, resulting from enhanced accumulation of reactive oxygen species (ROS) and impaired antioxidant capabilities in response to hyperglycemia, has been postulated as an important pathological mechanism, while the reduction of ROS attenuated the progression of various diabetic complications (8-13), including in the cornea (5; 14).

Substance P (SP), released predominantly by peripheral terminal, is an 11-amino acid neuropeptide which acts as a neurotransmitter mediating nociceptive transmission. It mainly functions through the interaction with neurokinin receptors, members of the tachykinin subfamily of G-protein-coupled receptors, among which the neurokinin-1 (NK-1) receptor shows a preferential affinity for SP. SP is currently known as a neuro-immunomodulatory regulator of the immune system (15). In the cornea, SP has been detected in the nerve fibers of naïve cornea (16; 17) and in antigen-presenting cells in herpetic stromal keratitis (18). Moreover, SP causes the mobilization of bone marrow-derived stem cells to participate in corneal wound healing (19; 20). Interestingly, recent evidences have shown that SP activates the EGFR, mitogen-activated protein kinases (MAPK), extracellular signal regulated kinases (ERK), and phosphoinositide 3-kinase-Akt (PI3K-Akt) signaling pathways, as well as promotes the healing of inflamed colonic epithelium (21) and possesses anti-apoptotic effects in colonocytes (22), dendritic cells (23), tenocytes (24), neutrophils (25), and bone marrow-derived stem cells (26).

The cornea is one of the most densely innervated tissues in the body, containing nerve fibers derived from the trigeminal ganglion. Corneal nerve fibers exert important trophic influences and
contribute to the maintenance of corneal epithelium homeostasis, whereas the dysfunction of corneal innervation produces an impairment of corneal epithelial wound healing, known as neurotrophic keratitis, as caused by for instance herpetic viral infections, trigeminal nerve damage, or diabetes mellitus (27-29). Although tear fluid impairment is also involved (30), the neurotrophic deficits may play a major role in the pathogenesis of diabetic keratopathy, as the density of corneal nerve fibers and corneal sensation decreased in diabetic patients (31-33). However, although being the major sensory neurotransmitter and neuropeptide released from corneal nerve fibers, SP was shown not to be significantly decreased in diabetic cornea (34). Nevertheless, paradoxically, topical SP application promoted corneal epithelial wound healing in diabetic animals and human when combined with insulin-like growth factor (IGF)-1 or epidermal growth factor (EGF) per group. The promoting mechanisms of SP on diabetic corneal epithelial wound healing remain elusive. In this study, we sought to demonstrate the protective mechanism of SP against diabetic corneal epithelial wound healing using streptozotocin-induced type 1 diabetic mice and high glucose-treated corneal epithelial cells.

**RESEARCH DESIGN AND METHODS**

**Animals**

Adult male C57BL/6 mice were purchased from the Beijing Pharmacology Institute (Beijing, China). All animal experiments were carried out in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research. The mice underwent induction of type 1 diabetes mellitus with an intraperitoneal injection of 50 mg/kg streptozotocin (STZ, Sigma, St. Louis, MO) in ice-cold citrate-citric acid buffer (pH 4.5) for 5 days, while control mice received equal amount of buffer. Blood glucose levels were monitored with an OneTouch Basic glucometer (LifeScan, Johnson & Johnson, Milpitas, CA). In the present study, diabetic mice were used after 12 weeks of final STZ injection, at which point the HbA1c values were 10.83±0.74% (94.75±7.93 mmol/mol), whereas normal mice had HbA1c values of 4.25±0.1% (22.5±1 mmol/mol). For topical SP application, 5 µl SP (1mM, Calbiochem, San Diego, CA) in distilled water were dropped on the corneal surface by a 10 µl tip, 6 times daily per eye for 4 days (for the measurement of corneal sensitivity) or 4 days pre-scrape and 3 days post-scrape (for the measurement of corneal epithelial wound healing). For NK-1 receptor inhibition, NK-1 receptor antagonist L-733,060 (6.6 µg in 5 µl distilled water, Sigma) was injected subconjunctivally 3 days before corneal
sensitivity measurement and corneal epithelium scrape in normal mice, or 24 h before corneal epithelium scrape in diabetic mice according to our preliminary experiments. Control mice were treated with distilled water vehicle.

**Corneal sensitivity**

Corneal sensation was measured bilaterally by using a Cochet-Bonnet esthesiometer (Luneau Ophtalmologie, Chartres Cedex, France) in unanesthetized control, diabetic, SP-treated diabetic mice, and the NK-1 receptor antagonist-injected mice before the scrape of corneal epithelium. The testing began with the maximal length (6 cm) nylon filament, and shortened by 0.5 cm each time until the corneal touch threshold was found. The longest filament length resulting in a positive response was considered as the corneal sensitivity threshold, which was verified 4 times.

**Corneal epithelial wound healing**

Normal, diabetic and SP-treated diabetic mice were anesthetized by an intraperitoneal injection of xylazine and ketamine followed by topical application of 2% xylocaine. The entire corneal epithelium including limbal region (marked with 3 mm trephine) was scraped with algerbrush II corneal rust ring remover (Alger Co, Lago Vista, TX) and subsequently applied with ofloxacin eye drops to avoid infection. Usually, one eye was wounded at a time in each animal. The defects of corneal epithelium were visualized at 24, 48 and 72 h by instilling 0.25% fluorescein sodium and photographed under slit lamp (BQ900, Haag-Streit, Bern, Switzerland). The staining area was analyzed by using Image J software and calculated as the percentage of residual epithelial defect.

**Corneal epithelial cell culture and treatment**

Mouse corneal epithelial cell line (TKE2) was presented from Dr. Tetsuya Kawakita of Keio University (Tokyo, Japan) (35). Human corneal tissues were handled according to the tenets of the Declaration of Helsinki. Primary human corneal epithelial cells (HCECs) were established from limbal explants of donor corneas according to a previous report (36). For the analysis of Akt-, EGFR- and Sirt1-signaling activation, and the staining of ROS, glutathione and mitochondria, both cells were starved overnight in bovine pituitary extract (BPE)-free keratinocyte serum-free medium (KSFM, Invitrogen, Carlsbad, CA) and subsequently incubated in 30 mM glucose or mannose (osmotic control) for 3 days with or without 1 µM SP.

**Cell proliferation and migration analysis**

For the proliferation analysis, the TKE2 cells were starved overnight in BPE-free KSFM, treated
with high glucose for 3 days in the absence or presence of 1 µM SP, and measured using MTT assay. For the migration analysis, the cells were cultured in high glucose until confluence, subsequently wounded with a micropipette tip, and incubated with or without SP for 24 h. Digital images of wound closure were used for quantitative assessment of migration by using Image J software. Each assay was conducted at least triplicate.

**Immunofluorescence staining**

Eyeballs were snap-frozen in Tissue-Tek optimum cutting temperature compound (Sakura Finetech, Tokyo, Japan). Frozen corneal sections were fixed in 4% paraformaldehyde for 15 min, permeabilized with 0.1% Triton X-100 for 10 min and blocked with normal serum for 1 h at room temperature. The samples were stained with primary antibodies overnight at 4°C, washed, and incubated with fluorescein-conjugated secondary antibodies at 37°C for 1 h (antibody information as listed in Table S1). All staining was observed under a confocal microscope or an Eclipse TE2000-U microscope (Nikon, Japan) after counterstained with 4',6-diamidino-2-phenylindole (DAPI).

**Reverse transcription quantitative-polymerase chain reaction**

Total RNA was extracted from mouse corneal epithelium using NucleospinRNA Kits (BD Biosciences, Palo Alto, CA). cDNAs were synthesized using the PrimeScript™ First-Strand cDNA Synthesis kit (TaKaRa, Dalian, China). Real time-PCR was carried out using SYBR® Green reagents and the Applied Biosystems 7500 Real Time PCR System (Applied Biosystems, Foster City, CA). The specific primers used are listed in Table S2. The cycling conditions were 10 sec at 95°C followed by 45 two-step cycles (15 sec at 95°C and 1 min at 60°C). The quantification data were analyzed with the Sequence Detection System software (Applied Biosystems) using GAPDH as an internal control.

**Western blot analysis**

Total protein was extracted from the lyzed samples of mouse corneal epithelium, cultured TKE2 cells and HCECs in RIPA buffer. Samples (total protein concentration: 40 µg for mouse corneal epithelium, 45 µg for cultured cells) were run on 12% SDS-PAGE gels and then transferred to a PVDF membrane (Millipore, Billerica, MA). The blots were blocked by non-fat dry milk for at least 1 h, and incubated with primary antibodies (Table S1) in TBST for 1 h at room temperature. The blots were washed three times and incubated with a HRP-conjugated secondary antibody (Amersham Biosciences, Piscataway, NJ). Finally, the blots were visualized via enzyme-linked chemiluminescence using the ECL kit (Chemicon, Temecula, CA).
Mitochondria superoxide and membrane potential staining

For the observation of mitochondrial structure, superoxide generation, and membrane potential, the cells were preloaded with 100 nM Mitotracker green (Beyotime, China) for 30 min, 5 µM MitoSOX\textsuperscript{TM} red reagent (Beyotime), and 5 µg/ml 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazole-carbocyanide iodine (JC-1; Beyotime), respectively, for 15 min at 37°C. The fluorescence was observed and captured using a Nikon confocal microscopy.

Measurement of intracellular ROS generation and glutathione content

For the observation of intracellular ROS and glutathione staining, fresh corneal cryostat sections and cultured cells were loaded with 10 µM fluorescence probe 2,7-dichlorodihydrofluorescein diacetate, acetyl ester (DCHF-DA; Molecular Probes, Eugene, OR) and 50 µM monochlorobimane (MCB, Sigma), respectively, for 30 min at 37°C. The staining was observed and captured using a Nikon confocal microscopy. For the measurement of ROS generation, 50,000 cells were harvested and incubated with 5 µM DCHF-DA for 20 min at 37°C. For the measurement of glutathione (GSH) content, 50,000 cells were harvested and freeze-thawed with liquid nitrogen and a 37°C water bath for 3 times. The supernatant was collected and mixed with the provided working buffer and NADPH. The total glutathione content was quantified by comparison with known glutathione standards according to the manufacturer instructions (Beyotime). The ROS and glutathione fluorescence intensity was measured using a MultiMode Microplate Reader (SpectraMax M2, Molecular Devices, Menlo Park, CA).

Statistical analysis

Data in this study were representative of at least three different experiments and presented as the means±SD. Statistical analysis was performed using SPSS 17.0 software (SPSS, Chicago, IL, USA) and one-way ANOVA (analysis of variance). Differences were considered statistically significant at $p<0.05$.

RESULTS

Substance P promotes corneal epithelial wound healing and sensitivity recovery in diabetic mice

To assess the effects of SP on diabetic corneal epithelial wound healing, entire corneal epithelium was scraped in age-matched normal mice and diabetic mice with or without topical SP application for 7 days. Punctate fluorescence staining showed that SP was detected in corneal epithelium after topical
application, which suggests the applied SP penetrated the apical tight junction barrier in diabetic mice (Fig. S1). The corneal epithelial healing rate exhibited a significant difference from 48 h after corneal epithelium scrape (Fig. 1A). The defect size of corneal epithelium in SP-treated diabetic mice (48 h: 28.78±11.78%; 72 h: 4.44±2.29%, n=6) was significantly improved from that of diabetic mice (48 h: 63.59±7.85%; 72 h: 22.73±9.85%, n=6), and reached the equal level of normal mice (48 h: 19.59±5.67%; 72 h: 3.08±2.17%, n=6) (Fig. 1B). Moreover, the attenuated corneal sensitivity in diabetic mice was also restored by topical SP application, although still being lower than that of normal mice (Fig. 1C, n=8 per group). In addition, compared with diabetic mice, more inflammatory cell infiltration was found underneath the corneal epithelium margin of SP-treated diabetic mice at 48 h, while reduced at 72 h after corneal epithelium scrape (Fig. S2). The results suggest that topical-applied SP penetrates into corneal epithelium and promotes corneal epithelial wound healing in diabetic mice, accompanied with recovery of corneal sensitivity, and an early inflammatory and resolution response.

**Substance P promotes corneal epithelial cell migration and proliferation in vitro**

To assess the effect of SP on corneal epithelial wound healing in vitro, mouse corneal epithelial cells were treated with high glucose with equal concentration of mannose as osmotic control. Subsequently, the confluent cells were wounded and treated with or without SP for another 24 h to analyze the migration rate. The results showed that high-glucose treatment caused significant delay of corneal epithelial cell migration, while SP addition improved the migration capacity of high glucose-treated cells to the same level of normal cells (Fig. 2A, B, n=3 per group). In addition, SP promoted the proliferation rate of corneal epithelial cells that was impaired by high-glucose treatment for 3 days (Fig. 2C, n=3 per group). The experiments were performed three times with similar results.

**Substance P reactivates Akt, EGFR and Sirt1 altered by hyperglycemia**

To elucidate the mechanism underlying the promotion of SP on corneal epithelial wound healing, we investigated the effects of SP on the activation of Akt, EGFR and Sirt1 that altered in diabetic corneal epithelium. Representative phosphorylated (p)-Akt, p-EGFR and Sirt1 staining is shown in Figure 3A. The expression levels of p-Akt, p-EGFR and Sirt1 were significantly up-regulated in diabetic corneal epithelium after topical SP application for 4 days (Fig. 3B, n=3 per group). Furthermore, SP also up-regulated the expression levels of p-Akt, p-EGFR and Sirt1 in both mouse TKE2 cells and human primary corneal epithelial cells that was impaired by high-glucose treatment (Fig. S3). The results suggest that SP application in both diabetic mice and cultured corneal epithelial
cells reactivates Akt, EGFR and Sirt1 that are altered by hyperglycemia, which may in part explain the promoting mechanisms of SP in diabetic corneal epithelial wound healing.

**Substance P attenuates oxidative stress of corneal epithelium by hyperglycemia**

To evaluate the effect of SP on the regulation of hyperglycemia-induced oxidative stress in corneal epithelium, corneal sections were loaded with fluorescence probe DCHF-DA and MCB for the detection of intracellular ROS and glutathione. Representative results showed that a significant increased ROS and reduced glutathione staining were detected in diabetic mouse corneal epithelium than in that of normal mice. However, a weak ROS and strong glutathione staining of corneal epithelium was found after topical SP application in diabetic mice (Fig. 4A). Moreover, the expression of major intracellular free radical scavengers in corneal epithelium, including manganese superoxide dismutase (MnSOD), catalase, NAD(P)H: quinone oxidoreductase 1 (NQO1), thioredoxin (TXN) and heme oxygenase 1 (Hmox1) in mRNA transcription level, were recovered from the diabetes mellitus after topical SP application (Fig. 4B, n=4 per group). In addition, immunofluorescence staining and Western blot revealed that the protein levels of NQO1, Catalase and MnSOD in diabetic corneal epithelium partially recovered after topical SP application (Fig. 4C, D, n=4 per group). The results suggest that topical SP application attenuates hyperglycemia-induced oxidative stress in diabetic corneal epithelium, at least via the mechanism of reducing ROS accumulation, and increasing intracellular glutathione content and antioxidant gene expression.

**Substance P attenuates mitochondrial dysfunction induced by high glucose**

Mitochondrial dysfunction plays an important role in the progress of various diabetic complications. To evaluate the effects of SP on the mitochondrial dysfunction in corneal epithelium induced by hyperglycemia, cultured corneal epithelial cells were treated with high glucose in the presence or absence of SP for 3 days. Exposure to elevated glucose caused increased ROS accumulation and reduced glutathione content of corneal epithelial cells in vitro (Fig. 5A, B), similar to the diabetic corneal epithelium in vivo. However, SP treatment significantly reduced the oxidative stress caused by high glucose in corneal epithelial cells, as showed by decreased ROS accumulation and increased glutathione content (Fig. 5A, B, n=3 per group). The results were also repeated by using the primary human corneal epithelial cells (Fig. S4). Furthermore, compared with normal or mannose-treated cells, high glucose-treated cells assumed apparent mitochondrial superoxide staining (MitoSox staining in Fig. 5C), accompanied with significant change of mitochondrial structure.
Mitotracker staining in Fig. 5C), and loss of mitochondrial membrane potential (red to green fluorescence of JC-1 staining in Fig. 5C). However, the addition of SP attenuated the generation of mitochondrial superoxide, and promoted the recovery of mitochondrial structure and membrane potential that were impaired by high-glucose treatment in corneal epithelial cells (Fig. 5C).

**The NK-1 receptor mediates the promotion of substance P on diabetic corneal epithelial wound healing**

To assess if the NK-1 receptor mediates the improvements of SP on diabetic corneal epithelial wound healing, the NK-1 receptor specific antagonist L-733,060 was injected before topical SP application in diabetic mice. In unwounded mouse corneal epithelium, the staining density of p-Akt, p-EGFR and Sirt1 were attenuated in antagonist-injected SP-treated diabetic mice, as compared to in the diabetic mice treated with SP alone (Fig. 6A). In corneal epithelium-scraped mice, the antagonist injection before SP application fully reversed the promotion of SP on diabetic corneal epithelial wound healing, with 28.95±3.89% epithelial defect in antagonist-injected SP-treated mice as compared to only 4.44±2.29% defect in mice treated with SP alone, and to 22.73±9.85% defect in untreated diabetic mice at 72 h (Fig. 6B, n=5 per group). Moreover, at 72 h post scrape, a stronger staining intensity of p-Akt and proliferation marker Ki-67 was found in the migrating area of SP-treated diabetic corneal epithelium than in that of either untreated or antagonist-injected diabetic corneal epithelium (Fig. 6C). The results suggest that the NK-1 receptor mediates the reactivation of Akt, EGFR, and Sirt1 by SP, and that the promotion of SP on diabetic corneal epithelial wound healing is also NK-1 receptor mediated.

**Local injection of NK-1 receptor antagonist causes diabetic pathological changes in normal mice**

To assess the effects of SP-NK-1 receptor signaling inhibition on corneal epithelium, L-733,060 was injected subconjunctivally in normal mice. After 3 days of antagonist injection, the unwounded corneal epithelium assumed a similar attenuation of p-Akt, p-EGFR, Sirt1 staining to that in diabetic mice (Fig. 7A), accompanied with decreased corneal sensitivity (Fig. 7B, n=5 per group). After 72 h of corneal epithelium scrape, the antagonist-injected mice showed a significant delay of corneal epithelial wound healing, with 31.77±5.07% epithelial defect in antagonist-injected mice as compared to only 3.08±2.17% defect in control mice (Fig. 7C, n=5 per group), and an attenuated p-Akt and Ki-67 staining in the migrating area of corneal epithelium as compared to that in normal mice (Fig. 7D), which were similar to the pathological changes in diabetic corneal epithelium (Fig. 7C, D). Similar
results were also obtained with the injection of another NK-1 receptor antagonist Spantide I (data not shown). The results show that local injection of NK-1 receptor antagonist in normal mice causes similar pathological changes in corneal epithelial wound healing and corneal sensitivity as that seen in diabetic mice, suggesting normal activation of SP-NK-1 receptor signaling plays a critical role in the homeostasis of corneal epithelium and corneal sensation.

**DISCUSSION**

The cornea is one of the most densely innervated parts of the human body, and as such its sensory nerves play not only a prominent role in nociception, but also in providing trophism to the corneal tissue. In diabetic mellitus, corneal sensitivity, nerve fiber density and epithelial wound healing is reduced significantly (27; 31; 32). However, the mechanisms are not completely understood. In the present study, we found that SP promoted epithelial wound healing, stimulated the reactivation of Akt, EGFR, and Sirt1, as well as attenuated oxidative stress in diabetic corneal epithelium. Furthermore, the study shows that the impairment of SP-NK-1 receptor signaling causes changes of normal corneal epithelium similar to those of diabetic mice. The results suggest that SP-NK-1 receptor signaling regulates the activation of multiple signaling pathways that are needed for the corneal epithelial wound healing, whereas this regulation is impaired in diabetic corneal epithelium and rescued by SP via an auto-regulatory mechanism (37; 38). Moreover, SP restored the corneal sensitivity of diabetic mice close to the same level of normal mice, whereas local inhibition of SP-NK-1 receptor signaling caused decreased corneal sensitivity in normal mice similar to that in diabetic mice. The results suggest that the impairment of SP-NK-1 receptor signaling may also be involved in the attenuation of corneal sensitivity in diabetes mellitus, which is supported by the fact that the NK-1 receptor exists in peripheral nerve (39). Taken together, SP, secreted by corneal sensory nerve fibers, may be the key neurotransmitter and neuropeptide that mediates corneal nociception transmission and provides trophism to the corneal epithelium. Furthermore, as for the treatment of diabetic keratopathy, many growth factors, cytokines and various agents have been evaluated as their capacity of accelerating corneal wound healing (40). However, SP and its functional derivative (FGLM-amide), with the advantages of smaller molecules and higher efficiency, have been shown to be effective for the treatment of persistent corneal epithelial defects in clinical studies (40-42). In addition, we found that SP promoted the regeneration of nerve fibers in diabetic corneal epithelium and accelerated trigeminal
neuronal growth in vitro that impaired by high glucose (Fig. S5).

The NK-1 receptor, the preferred receptor of SP, mediates a variety of physiological and pathophysiological responses (22; 23; 43). Although previous studies have confirmed that SP enhances corneal epithelial migration when combined with IGF-1 or EGF (41; 44; 45), the exact mechanism remains unclear. Here we show that SP reactivates the Akt, EGFR and Sirt1, and promotes ROS scavenging capacity that is impaired by hyperglycemia. The results suggest a molecular basis for the synergistic effects of SP and IGF-1 or EGF on the enhancement of diabetic corneal epithelial wound healing (46; 47). Even more interestingly, we found that the local inhibition of the SP-NK-1 receptor signaling in normal mice causes pathological changes of the corneal epithelium similar to those of diabetic corneal epithelium. These results suggest that SP-NK-1 receptor signaling may be involved in the maintenance of corneal epithelium homeostasis, and also in the protection from hyperglycemia stress, whereas the impairment of SP-NK-1 receptor signaling may explain the fragility of diabetic corneal epithelium in vivo.

Prolonged hyperglycemia always causes the perturbation of catabolic pathways and the over-production of ROS in the mitochondria, which in turn plays a critical role in the development of diabetic complications (48), including diabetic keratopathy (5). In the present study, we confirmed that the mitochondrial superoxide level was up-regulated in high glucose-treated corneal epithelial cells, accompanied by changes of mitochondria structure and loss of mitochondrial membrane potential. Interestingly, we found that SP attenuates the dysfunction of the mitochondria by high glucose. Moreover, SP also elevates the intracellular glutathione level, the main antioxidant in the cells. In addition, although increased Nrf2 expression was detected in diabetic corneal epithelium (data not shown), the expressions of Nrf2 downstream antioxidant genes, including MnSOD, catalase, NQO1, TXN and Hmox1, were down-regulated in diabetic corneal epithelium, while up-regulated after SP application, which suggests that additional regulatory mechanisms may exist between Nrf2 and its downstream antioxidant gene expressions in diabetes mellitus (49; 50). All things considered, the improvement of oxidative stress by SP via the improved mitochondrial function, elevated GSH level and up-regulated expression of antioxidant genes may also plays an important role in the protection of corneal epithelium in diabetes mellitus.

In conclusion, our study demonstrates, for the first time, that SP promotes diabetic corneal epithelial wound healing while simultaneously triggering the reactivation of the Akt-, EGFR- and
Sirt1-signaling of importance for that healing, as well as rescuing corneal sensation, improving mitochondrial function, and decreasing ROS accumulation impaired by hyperglycemia. We furthermore show that local inhibition of SP-NK-1 receptor signaling abolishes the promotion of SP on corneal epithelial healing in diabetic mice, and causes diabetic corneal pathological changes in normal mice. Thus, the SP-NK-1 receptor signaling may play a critical role in the maintenance of corneal epithelium homeostasis, and SP signaling may, through the NK-1 receptor, contribute to the promotion of diabetic corneal epithelial wound healing by the rescued activation of Akt, EGFR and Sirt1, the improvement of mitochondrial function, and the increased ROS scavenging capacity of corneal epithelium.

ACKNOWLEDGMENTS

This work was partially supported by the National Basic Research Program of China (2012CB722409) and the National Natural Science Foundation of China (81170816, 81200665). Qingjun Zhou is partially supported by the Shandong Provincial Excellent Innovation Team Program and Taishan Scholar Program (20081148). Patrik Danielson is partially supported by the J.C. Kempe and Seth M. Kempe Memorial Foundations, the Swedish Society of Medicine, the Cronqvist and KMA foundations, and the National Swedish Research Council (521-2013-2612; Q. Zhou co-applicant). The authors thank Yangyang Zhang, Wenjie Sui, Qian Wang, Hua Gao, Suxia Li and Zhaoli Chen of Shandong Eye Institute for their help with the animal experiments, statistical analysis, and human tissue collection.

No potential conflicts of interest relevant to this article were reported.

L.Y and G. D contributed to sample testing, data analysis and study design; X. Q, M. Q. Y. W, and H. D contributed to samples testing and data analysis; P. D, L. X and Q. Z contributed to study design, data analysis and manuscript preparation. L. X and Q. Z are the guarantors of this work and, as such, have full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.
REFERENCES


38. Goode T, O'Connor T, Hopkins A, Moriarty D, O'Sullivan GC, Collins JK, O'Donoghue D, Baird AW, O'Connell J, Shanahan F: Neurokinin-1 receptor (NK-1R) expression is induced in human colonic...
epithelial cells by proinflammatory cytokines and mediates proliferation in response to substance P. J Cell Physiol 197:30-41, 2003


FIGURE LEGENDS

Figure 1. Substance P promotes corneal epithelial wound healing and restores corneal sensitivity in diabetic mice. Topical SP application for 7 days was used to examine the wound healing rate in diabetic corneal epithelium. The corneal epithelial wound was inflicted after 4 days of SP application and then stained with fluorescein sodium at 24, 48 and 72 h after the corneal epithelium scrape, with continuous daily SP administration in the ‘Diabetic+SP’-group (A). Histogram of residual epithelial defect is presented as the percentage of the original wound (B, n=6 per group). Corneal sensitivity was measured in unanesthetized control, diabetic and SP-treated diabetic mice after 4 days’ topical application (C, n=8 per group). * p < 0.05, ns: no significance.

Figure 2. Substance P promotes the migration and proliferation of corneal epithelial cells impaired by high glucose. Confluent mouse corneal epithelial cells were wounded after the treatment with 30 mM glucose or mannose for 3 days. Cell migration was observed with or without SP treatment for another 24 h (A), and the migration area was analyzed by Image J software (B, n=3 per group). Cell proliferation was measured using MTT assay after the treatment for 3 days with glucose or mannose in the absence or presence of SP (C, n=3 per group). * p < 0.05, ns: no significance.

Figure 3. Substance P reactivates Akt, EGFR and Sirt1 in diabetic corneal epithelium. Topical SP application for 4 days was used to examine the reactivation of Akt, EGFR and Sirt1 in diabetic corneal epithelium. SP recovered the positive staining (A) and up-regulated the protein levels of phosphorylated (p)-Akt, p-EGFR and Sirt1 in diabetic corneal epithelium, as compared to that of untreated diabetic mice (B, n=3 per group). * p < 0.05, ns: no significance.

Figure 4. Substance P attenuates oxidative stress of diabetic corneal epithelium. Topical SP application for 4 days was used to examine the attenuation of oxidative stress in diabetic corneal epithelium. SP restored the ROS and glutathione (GSH) staining in diabetic corneal epithelium to similar levels as those in normal corneal epithelium (A). SP elevated the mRNA transcript levels of antioxidant genes MnSOD, Catalase, NQO1, GCLC, TXN and Hmox1 (B, n=4 per group), as
well as the staining density and protein levels of NQO1, Catalase and MnSOD in diabetic corneal epithelium, as compared to that in untreated diabetic corneal epithelium (C, D, n=4 per group). *p < 0.05, ns: no significance.

**Figure 5. Substance P improves mitochondrial functions of corneal epithelial cells triggered by high glucose.** Mouse corneal epithelial cells were treated with 30 mM glucose or mannose for 3 days in the presence or absence of SP. SP recovered the staining density and levels of intracellular ROS and glutathione (GSH) (A, B, n=3 per group). SP improved the impaired mitochondrial functions by high glucose (C), including the mitochondrial superoxide (MitoSox staining), mitochondrial structure (Mitotacker staining) and mitochondrial membrane potential (JC-1 staining). *p < 0.05.

**Figure 6. NK-1 receptor antagonist blocks the promotion of substance P on diabetic corneal epithelial wound healing.** NK-1 receptor antagonist L-733,060 was injected subconjunctivally at 24 h before topical SP application in diabetic mice. In the unwounded corneal epithelium, the elevation of p-Akt, p-EGFR and Sirt1 staining density by SP application was attenuated in antagonist-injected SP-treated diabetic mice (A). In the corneal epithelium 72 h after scrape, the antagonist injection reversed the promotion of SP on diabetic corneal epithelial wound healing (B, n=5 per group) and the staining intensity of p-Akt and the proliferation marker Ki-67 in the regenerated corneal epithelium (C). *p < 0.05, ns: no significance.

**Figure 7. Local inhibition of SP-NK-1 receptor signaling causes diabetic-like pathological changes in the cornea of normal mice.** NK-1 receptor antagonist L-733,060 was injected subconjunctivally in normal mice. In the unwounded corneal epithelium of 3 days after antagonist injection, the staining density of p-Akt, p-EGFR and Sirt1 (A) and corneal sensitivity (B, n=5 per group) was attenuated similarly to that in diabetic mice. In the corneal epithelium of 72 h after scrape, the local antagonist injection caused a significant delay of corneal epithelial wound healing (C, n=5 per group) as well as attenuated p-Akt and Ki-67 staining density in the regenerated corneal epithelium (D). *p < 0.05, ns: no significance.
ONLINE SUPPLEMENTAL MATERIALS

Figure S1. Topically applied substance P penetrates into the corneal epithelium in diabetic mice. SP was detected in the cytoplasm of corneal epithelial cells by immunofluorescence staining, which suggests that topically applied SP can penetrate into the corneal epithelium in diabetic mice.

Figure S2. Substance P promotes the early inflammatory and resolution response in diabetic cornea. At 48 h after corneal epithelium scrape, more inflammatory cell infiltration was found underneath the corneal epithelium margin of SP-treated diabetic mice as compared to untreated diabetic mice, whereas this response was reduced at 72 h when compared with diabetic mice (black lines represent the basal of regenerating corneal epithelium).

Figure S3. Substance P recovers p-Akt, p-EGFR and Sirt1 levels impaired by high glucose in cultured corneal epithelial cells. Mouse TKE2 (A) cells or human primary corneal epithelial cells (B) were treated with high glucose in the absence or presence of SP for 3 days, with mannose as osmotic control. SP recovered the expression levels of p-Akt, p-EGFR and Sirt1 that was impaired by high-glucose treatment in both mouse TKE2 cells (A, n=4 per group) and primary human corneal epithelial cells (B, n=3 per group). * p < 0.05, ns: no significance.

Figure S4. Substance P attenuates oxidative stress of primary human corneal epithelial cells induced by high glucose. Human primary corneal epithelial cells were treated with 30 mM glucose or mannose for 3 days in the presence or absence of SP. SP recovered the staining density of intracellular ROS and glutathione (GSH).

Figure S5. Substance P promotes the regeneration of diabetic corneal nerve fibers in vivo and the growth of high glucose-treated trigeminal ganglion cells in vitro. To explore the promotion of SP on corneal nerve fiber regeneration in diabetic mice, central corneal epithelium (marked with 2 mm trephine) was scraped after 6 times of topical SP application per day for 4 days. The SP application was continued with 6 times per day for another 3-4 days until the completion of corneal epithelial wound healing, subsequently with 1-2 times per day for 2 weeks until sample collection. The whole-mounted corneal staining with β3-tubulin antibody demonstrated that SP
application significantly promoted the regeneration of nerve fibers in diabetic corneal epithelium (A, C, n=3 per group). *In vitro*, mouse trigeminal nerves were isolated and incubated in Neurobasal A medium as previous descriptions [1]. The fresh-isolated trigeminal neuronal cells were treated with 30 mM glucose for 3 days with or without 1 µM SP. The results demonstrated that SP accelerated neuronal growth that was impaired by high glucose, as showed by the immunofluorescence staining with β3-tubulin antibody (B) and the counting of total nerve fiber length (D, n=6 per group). *p < 0.05, ns: no significance.

References

Figure 1. Substance P promotes corneal epithelial wound healing and restores corneal sensitivity in diabetic mice. Topical SP application for 7 days was used to examine the wound healing rate in diabetic corneal epithelium. The corneal epithelial wound was inflicted after 4 days of SP application and then stained with fluorescein sodium at 24, 48 and 72 h after the corneal epithelium scrape, with continuous daily SP administration in the 'Diabetic+SP'-group (A). Histogram of residual epithelial defect is presented as the percentage of the original wound (B, n=6 per group). Corneal sensitivity was measured in unanesthetized control, diabetic and SP-treated diabetic mice after 4 days' topical application (C, n=8 per group). * p < 0.05, ns: no significance.
Figure 2. Substance P promotes the migration and proliferation of corneal epithelial cells impaired by high glucose. Confluent mouse corneal epithelial cells were wounded after the treatment with 30 mM glucose or mannose for 3 days. Cell migration was observed with or without SP treatment for another 24 h (A), and the migration area was analyzed by Image J software (B, n=3 per group). Cell proliferation was measured using MTT assay after the treatment for 3 days with glucose or mannose in the absence or presence of SP (C, n=3 per group). * p < 0.05, ns: no significance.
Figure 3. Substance P reactivates Akt, EGFR and Sirt1 in diabetic corneal epithelium. Topical SP application for 4 days was used to examine the reactivation of Akt, EGFR and Sirt1 in diabetic corneal epithelium. SP recovered the positive staining (A) and up-regulated the protein levels of phosphorylated (p)-Akt, p-EGFR and Sirt1 in diabetic corneal epithelium, as compared to that of untreated diabetic mice (B, n=3 per group). * p < 0.05, ns: no significance.
Figure 4. Substance P attenuates oxidative stress of diabetic corneal epithelium. Topical SP application for 4 days was used to examine the attenuation of oxidative stress in diabetic corneal epithelium. SP restored the ROS and glutathione (GSH) staining in diabetic corneal epithelium to similar levels as those in normal corneal epithelium (A). SP elevated the mRNA transcript levels of antioxidant genes MnSOD, Catalase, NQO1, GCLC, TXN and Hmox1 (B, n=4 per group), as well as the staining density and protein levels of NQO1, Catalase and MnSOD in diabetic corneal epithelium, as compared to that in untreated diabetic corneal epithelium (C, D, n=4 per group). * p < 0.05, ns: no significance.

88x119mm (300 x 300 DPI)
Figure 5. Substance P improves mitochondrial functions of corneal epithelial cells triggered by high glucose. Mouse corneal epithelial cells were treated with 30 mM glucose or mannose for 3 days in the presence or absence of SP. SP recovered the staining density and levels of intracellular ROS and glutathione (GSH) (A, B, n=3 per group). SP improved the impaired mitochondrial functions by high glucose (C), including the mitochondrial superoxide (MitoSox staining), mitochondrial structure (Mitotracker staining) and mitochondrial membrane potential (JC-1 staining). * p < 0.05.

88x155mm (300 x 300 DPI)
Figure 6. NK-1 receptor antagonist blocks the promotion of substance P on diabetic corneal epithelial wound healing. NK-1 receptor antagonist L-733,060 was injected subconjunctivally at 24 h before topical SP application in diabetic mice. In the unwounded corneal epithelium, the elevation of p-Akt, p-EGFR and Sirt1 staining density by SP application was attenuated in antagonist-injected SP-treated diabetic mice (A). In the corneal epithelium 72 h after scrape, the antagonist injection reversed the promotion of SP on diabetic corneal epithelial wound healing (B, n=5 per group) and the staining intensity of p-Akt and the proliferation marker Ki-67 in the regenerated corneal epithelium (C). * p < 0.05, ns: no significance.
Figure 7. Local inhibition of SP-NK-1 receptor signaling causes diabetic-like pathological changes in the cornea of normal mice. NK-1 receptor antagonist L-733,060 was injected subconjunctivally in normal mice. In the unwounded corneal epithelium of 3 days after antagonist injection, the staining density of p-Akt, p-EGFR and Sirt1 (A) and corneal sensitivity (B, n=5 per group) was attenuated similarly to that in diabetic mice. In the corneal epithelium of 72 h after scrape, the local antagonist injection caused a significant delay of corneal epithelial wound healing (C, n=5 per group) as well as attenuated p-Akt and Ki-67 staining density in the regenerated corneal epithelium (D). * p < 0.05, ns: no significance.

177x118mm (300 x 300 DPI)
Figure S1. Topically applied substance P penetrates into the corneal epithelium in diabetic mice. SP was detected in the cytoplasm of corneal epithelial cells by immunofluorescence staining, which suggests that topically applied SP can penetrate into the corneal epithelium in diabetic mice.

21x17mm (300 x 300 DPI)
Figure S2. Substance P promotes the early inflammatory and resolution response in diabetic cornea. At 48 h after corneal epithelium scrape, more inflammatory cell infiltration was found underneath the corneal epithelium margin of SP-treated diabetic mice as compared to untreated diabetic mice, whereas this response was reduced at 72 h when compared with diabetic mice (black lines represent the basal of regenerating corneal epithelium).

88x42mm (300 x 300 DPI)
Figure S3. Substance P recovers p-Akt, p-EGFR and Sirt1 levels impaired by high glucose in cultured corneal epithelial cells. Mouse TKE2 (A) cells or human primary corneal epithelial cells (B) were treated with high glucose in the absence or presence of SP for 3 days, with mannose as osmotic control. SP recovered the expression levels of p-Akt, p-EGFR and Sirt1 that was impaired by high-glucose treatment in both mouse TKE2 cells (A, n=4 per group) and primary human corneal epithelial cells (B, n=3 per group). * p < 0.05, ns: no significance.

88x104mm (300 x 300 DPI)
Figure S4. Substance P attenuates oxidative stress of primary human corneal epithelial cells induced by high glucose. Human primary corneal epithelial cells were treated with 30 mM glucose or mannose for 3 days in the presence or absence of SP. SP recovered the staining density of intracellular ROS and glutathione (GSH).

88x49mm (300 x 300 DPI)
Figure S5. Substance P promotes the regeneration of diabetic corneal nerve fibers in vivo and the growth of high glucose-treated trigeminal ganglion cells in vitro. To explore the promotion of SP on corneal nerve fiber regeneration in diabetic mice, central corneal epithelium (marked with 2 mm trephine) was scraped after 6 times of topical SP application per day for 4 days. The SP application was continued with 6 times per day for another 3-4 days until the completion of corneal epithelial wound healing, subsequently with 1-2 times per day for 2 weeks until sample collection. The whole-mounted corneal staining with β3-tubulin antibody demonstrated that SP application significantly promoted the regeneration of nerve fibers in diabetic corneal epithelium (A, C, n=3 per group). In vitro, mouse trigeminal nerves were isolated and incubated in Neurobasal A medium as previous descriptions [1]. The fresh-isolated trigeminal neuronal cells were treated with 30 mM glucose for 3 days with or without 1 µM SP. The results demonstrated that SP accelerated neuronal growth that was impaired by high glucose, as showed by the immunofluorescence staining with β3-tubulin antibody (B) and the counting of total nerve fiber length (D, n=6 per group). * p < 0.05, ns: no significance.

References
Supplementary Table 1: Primary antibodies for immunofluorescent staining and Western blots

<table>
<thead>
<tr>
<th>Primary antibody</th>
<th>Residue</th>
<th>Supplier</th>
<th>Code</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substance P</td>
<td></td>
<td>Santa cruz</td>
<td>sc9758</td>
</tr>
<tr>
<td>p-AKT</td>
<td>pS473</td>
<td>Epitomics</td>
<td>2118-1</td>
</tr>
<tr>
<td>AKT</td>
<td>-</td>
<td>Epitomics</td>
<td>1085-1</td>
</tr>
<tr>
<td>p-EGFR</td>
<td>pY1068</td>
<td>Epitomics</td>
<td>1727-1</td>
</tr>
<tr>
<td>EGFR</td>
<td>-</td>
<td>Epitomics</td>
<td>1902-1</td>
</tr>
<tr>
<td>Sirt1</td>
<td>-</td>
<td>AbCam</td>
<td>Ab12193</td>
</tr>
<tr>
<td>NQO1</td>
<td>C-terminus</td>
<td>AbCam</td>
<td>Ab34173</td>
</tr>
<tr>
<td>Catalase</td>
<td>-</td>
<td>AbCam</td>
<td>Ab16731</td>
</tr>
<tr>
<td>MnSOD</td>
<td>-</td>
<td>AbCam</td>
<td>Ab13533</td>
</tr>
<tr>
<td>Ki-67</td>
<td></td>
<td>Abcam</td>
<td>Ab15580</td>
</tr>
<tr>
<td>γ3-tubulin</td>
<td></td>
<td>R&amp;D</td>
<td>NL1195R</td>
</tr>
<tr>
<td>Alexa Fluor 488 donkey anti-rabbit IgG</td>
<td>Life technologies</td>
<td>A21206</td>
<td></td>
</tr>
<tr>
<td>Alexa Fluor 594 donkey anti-rabbit IgG</td>
<td>Life technologies</td>
<td>A21207</td>
<td></td>
</tr>
<tr>
<td>Donkey anti-goat IgG-CFL 488</td>
<td>Santa cruz</td>
<td>sc362255</td>
<td></td>
</tr>
<tr>
<td>Gene</td>
<td>Accession number</td>
<td>Forward primer</td>
<td>Reverse primer</td>
</tr>
<tr>
<td>--------</td>
<td>------------------</td>
<td>------------------</td>
<td>------------------</td>
</tr>
<tr>
<td>MnSOD</td>
<td>NM_013671.3</td>
<td>GTGGAGAACCCAAAAAGGAGAG</td>
<td>AACCCTGGGACTCCACAGAC</td>
</tr>
<tr>
<td>Catalase</td>
<td>NM_009804.2</td>
<td>GTCCTCGTCCTCCAGGTCTTCTTCT</td>
<td>CTGCCTCTCCACATGTCATTA</td>
</tr>
<tr>
<td>NQO1</td>
<td>NM_008706.5</td>
<td>GCCCCTCAGAAGACCCAT</td>
<td>TTTGGGAGGAGGACCTTAAA</td>
</tr>
<tr>
<td>GCLC</td>
<td>NM_010295.2</td>
<td>CAGCAGTGTGCTCATCTTCTTT</td>
<td>TTTGGGAGGAGGACCTTAAA</td>
</tr>
<tr>
<td>TXN</td>
<td>NM_011660.3</td>
<td>TGTTGGACTCCCTCTTCTTCTTCTA</td>
<td>CTTCCAGTCTCCAGCAACA</td>
</tr>
<tr>
<td>Hmox1</td>
<td>NM_010442.2</td>
<td>GCACTAGCTCTCCACAGCA</td>
<td>CATGGCATAAATTCCACCTG</td>
</tr>
<tr>
<td>GLRX</td>
<td>NM_053108.4</td>
<td>CCTCGTGCACTGCTTTTCA</td>
<td>CTCCGGTGAGGCTGTTGAAAA</td>
</tr>
</tbody>
</table>